TRANSLATIONAL STUDIES IN OLDER MEN USING TESTOSTERONE TO TREAT SARCOPENIA

RANDALL J. URBAN, and (*by invitation*) E. L. DILLON, S. CHOUDHARY, Y. ZHAO, A. M. HORSTMAN, R. G. TILTON, and M. SHEFFIELD-MOORE

GALVESTON, TEXAS

ABSTRACT

Sarcopenia is the loss of skeletal muscle mass and strength that occurs with aging. Our research group has found an efficacious administration paradigm using testosterone to combat sarcopenia in humans. In addition, our research has uncovered an important regulatory enzyme of inflammation, nuclear factor–κB–inducing kinase that may regulate human skeletal muscle catabolism, and that appears to be counter-regulated by administration of standard doses of testosterone. This is important because a number of age-related clinical circumstances trigger acute and chronic muscle loss including cancer, chronic obstructive pulmonary disease, hospitalization, acute and chronic illness, and diseases in which systemic inflammation occurs. Moreover, it is often the treatment itself that can induce muscle loss. For example, glucocorticoids are tremendously effective at reducing inflammation and are a frontline therapy for many inflammatory-based diseases, yet paradoxically trigger muscle loss. We will discuss our research findings and the clinical significance of our human clinical translational research with testosterone.

INTRODUCTION

Aging is associated with decreased skeletal muscle insulin sensitivity, decreased anabolic responses to nutritional intake, and an overall decrease in muscle mass and function. Although healthy aging is not necessarily associated with increased skeletal muscle catabolism (1), the aging process has been associated with increased systemic low-grade inflammation (2, 3), increased hypothalamic-pituitary-adrenal stress responses (4–6), and in men, decreased basal hypothalamic-pituitary-gonadal response and decreased testosterone production (7, 8). These changes may contribute towards a metabolic tipping point of

Correspondence and reprint requests: Randall J. Urban, MD, The University of Texas Medical Branch, 301 University Boulevard, Galveston, TX 77555-0569, Tel: 409-772-1176, Fax: 409-772-8762, E-mail: rurban@utmb.edu.

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increased susceptibility for muscle wasting in response to catabolic triggers due to metabolic stress or disease.

Muscle wasting that occurs with sarcopenia can occur in a rapid, acute manner or more gradually in a chronic fashion and is often driven by stimulation of inflammatory pathways and downstream activation of transcription factors such as nuclear factor-κB (NF-κB) (9, 10). NF-κB signaling plays a prominent role in skeletal muscle atrophy, and pro-inflammatory cytokines induce skeletal muscle atrophy through downstream signaling requiring RelA/p65 (10). NF-κB–inducing kinase (NIK) is an upstream NF-κB pathway activating kinase, tightly regulated by cells to maintain low basal levels. Recent reports have shown significantly elevated steady-state NIK levels in diabetic kidneys (11) and other degenerative conditions, including multiple myeloma (12) and inflammatory arthritis (13).

Hypogonadism affects up to 4.5 million men in the United States (14) and further increases risk for comorbidities (15). Clinicians in the United States have taken note and prescriptions of androgens such as testosterone to men 40 years and older have increased more than 3-fold in the last decade (16). Androgen replacement therapy is traditionally prescribed clinically to replace low levels of endogenous serum testosterone and to combat the accompanying side effects of low androgens, including loss of skeletal muscle mass and strength. The anabolic benefits of androgens on skeletal muscle mass are well-documented (17–29). Furthermore, androgens, such as testosterone, suppress skeletal muscle catabolism in men (30, 31), and dehydroepiandrosterone (DHEA), a metabolic precursor to testosterone, has anti-inflammatory properties in peripheral tissues (32). However, a clear understanding behind the mechanism through which androgens regulate skeletal muscle protein catabolism is still elusive and could provide an important clinical target for therapies aimed at fighting muscle wasting diseases.

In vitro studies have shown that testosterone affects myogenesis and increases local insulin-like growth factor–1 (IGF-1) expression (33), regulates forkhead box O (FOXO1), peroxisome proliferator-activated receptor-gamma coactivator 1 alpha (PGC-1α), and p38 mitogen-activated protein kinases (MAPK) (34), and stimulates hypertrophy of L6 myoblasts that occurs via the androgen receptor and results in a signaling cascade dependent upon Erk and mammalian target of rapamycin (mTOR) (35). Although the anabolic effects of testosterone on skeletal muscle are thought to be mediated via androgen receptors expressed in myonuclei and satellite cells (36), the mechanisms behind the anti-catabolic effects of testosterone on human skeletal muscle have not been elucidated. In rat skeletal muscle, the anti-catabolic

actions of testosterone have been described through the repression of atrogin-1 and muscle RING-finger protein-1 (MuRF-1) expression (37).

In this paper, we have postulated that aging is associated with increased basal skeletal muscle NIK levels, and that androgens may be protective against skeletal muscle catabolism by suppressing the accumulation of NIK. We have examined the relationship between age, testosterone, and skeletal muscle NIK content in vivo and in vitro, and we are reporting that testosterone treatment in older men with low normal endogenous testosterone levels is capable of decreasing skeletal muscle NIK levels. We are also reporting that glucocorticoid-induced increases in NIK expression in human primary skeletal muscle cells are blocked in the presence of testosterone.

METHODS

Ethics Statement

The study was approved by The University of Texas Medical Branch (UTMB) Institutional Review Board and complied with the Declaration of Helsinki. Written informed consent was obtained from all subjects.

Participants and Study Design

In experiment 1, skeletal muscle tissue was obtained from consenting younger and older adults; either during baseline sample collection during ongoing clinical studies at the UTMB Institute of Translational Sciences Clinical Research Center (ITS CRC) (2 younger, 3 older) or a kind gift from Partha Sarkar, UTMB, Galveston (3 younger, 2 older). In experiment 2, healthy older men (67 $\pm$ 5 years, n = 6) with endogenous testosterone concentrations in the lower half of the normal range (200–500 ng/dL) volunteered to participate in a randomized open-label experiment through the UTMB ITS CRC. Participants were randomized to receive testosterone as either a single intramuscular injection (testosterone enanthate, 100 mg, n = 3) or as a gel to be applied daily to the skin (1% AndroGel [AbbVie Inc., North Chicago, IL], 10 g gel containing 100 mg testosterone each day, n = 3). Plasma and skeletal muscle tissue were collected before the first treatment and again after 7 days. Subjects were instructed to refrain from making changes to their habitual dietary intake and level of physical activity during this week. All subjects in experiment 2 reported to the CRC on a daily basis and subjects in the topical gel group received daily treatment under

nurse supervision to ensure proper application. In the experiment 3, human primary skeletal muscle cells and C2C12 cells were used for the in vitro experiments.

Human Skeletal Muscle Biopsy Procedure

Skeletal muscle tissue was collected from the lateral portion of the vastus lateralis (38). Briefly, a site was marked approximately 10 to 15 cm above the knee and the area was cleaned with Betadine (Medline Industries, Inc., Mundelein, IL). Lidocaine (Hospira, Inc., Lake Forest, IL) was applied to the skin and muscle to anesthetize the local area. An approximately 5- to 6-mm incision was made through the skin and fascia, and muscle tissue was collected using a 5-mm Bergström biopsy needle while suction was applied. The incision was sutured or sealed with Dermabond. Muscle tissue collected for molecular assays was immediately blotted, frozen in liquid nitrogen, and stored in a −80° C freezer until time of analysis. Fresh tissue for primary cell culture (from experiment 3) was collected in transfer buffer (Ca^{2+} and Mg^{2+} free Hanks Balanced Salt Solution; Invitrogen) and immediately processed.

Tissue Culture Using Human Primary Skeletal Muscle and C2C12 Cells

Satellite cells from human skeletal muscle biopsy samples were isolated and cultured as described earlier with some modification (39). Briefly, freshly obtained muscle biopsy sample (0.05 to 0.2g) was digested by dispase II and collagenase for 40 minutes at 37° C, and then filtered through 40-μm cell strainer. The filtered cells were centrifuged and resuspended in growth medium (Hams F10 media + 20% FBS + βFGF (5 ng/mL final) + 1% pen/strep + 0.1% gentamicin. The cell suspension was transferred to a 60-mm culture dish and left undisturbed for 48 to 72 hours in tissue culture incubator. Thereafter, fresh media was added every 2 days. Similarly, C2C12 cells were cultured in complete growth medium containing Dulbecco's modified eagle medium with 10% fetal bovine serum and 1% pen/strep and incubated in the atmosphere of 5% CO_2 at 37° C. The medium was changed every 2 or 3 days.

Glucocorticoids are known to induce protein catabolism (40) and testosterone inhibits glucocorticoid-induced protein catabolism in C2C12 cells (41). Therefore, we explored the use of physiological concentrations of methylprednisolone (Medrol), a commonly prescribed glucocorticoid in humans, to stimulate protein catabolism in human

primary muscle cell cultures and differentiated C2C12 cells. Changes in the expression of NIK mRNA and protein were determined before and after treatment with methylprednisolone. Primary cultures of skeletal myoblasts were differentiated for 4 days before treating with methylprednisolone (0.5 μg/mL) for 24 hours. To determine the effects of androgens on the regulation of NIK expression, testosterone was added at 1 μM concentration for 0, 3, and 6 hours.

Western Immunoblotting

Proteins were fractionated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis and transferred to nitrocellulose or polyvinylidene difluoride membranes (Millipore, North Bend, OH, USA). Membranes were blocked in 5% milk or 5% bovine serum albumin for 1 hour, and then incubated with the indicated primary antibody at 4° C overnight. Membranes were washed in Tris-buffered saline, 0.1% Tween-20, and incubated with secondary antibody at 20° C for 1 hour. Signals were visualized on a chemiluminescence detection film using the ECL (GE Healthcare, Piscataway, NJ, USA) system. β-actin was used as a loading control. Each target protein was quantified using the ImageJ software. NIK antibody was purchased from Cell Signaling, whereas antibodies against MuRF1 and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were obtained from Santa Cruz Biotechnology. The monoclonal antibody against β-actin was procured from Sigma-Aldrich.

Quantitative Real-Time Polymerase Chain Reaction

Cellular RNA was extracted using Tri Reagent (Sigma). RNA was quantified by Nanodrop (Thermo Scientific, Waltham, MA) and samples were included when the 260/280 nm ratio was > 1.6. A total of 2 μg RNA was used for reverse transcription using the SuperScript III First-Strand Synthesis System from Invitrogen (Carlsbad, CA). Real-time polymerase chain reactions (PCRs) were performed in triplicate, and a total of 2 μL cDNA products was amplified in a 20-μL reaction system containing 10 μL iQ SYBR Green Supermix (Bio-Rad) and 400 nmol/L primer mixture. Relevant primers were purchased from SA Bioscience (Frederick, MD). All reactions were processed in a MyiQ Single Color Real-Time PCR thermocycler using a two-step–plus–melting curve program. Results were analyzed by the iQ5 program (Bio-Rad), and the data were analyzed using the $\Delta\Delta$CT method in reference to GAPDH.

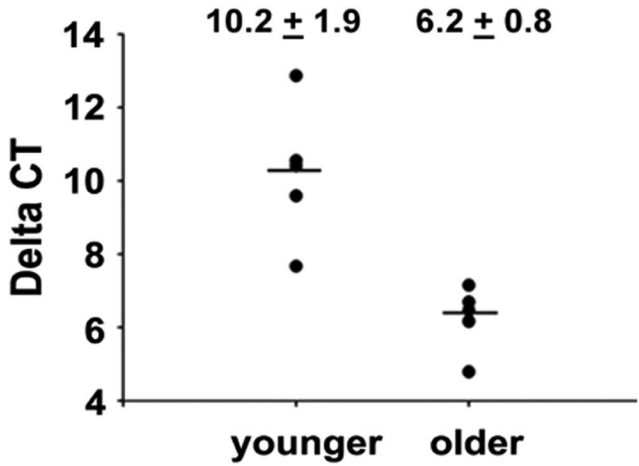

FIG. 1. Increased muscle NIK mRNA levels in old subjects. Human vastus lateralis muscle was sampled from young (29 ± 6 years) vs older (67 ± 5 years) subjects and assayed for NIK expression using qRT-PCR, with data analyzed using the delta CT method with GAPDH as the reference standard (lower delta CT means higher mRNA expression).

Statistics

All results are expressed as means ± SEM. Data from the older subjects (ie, injection or gel) were combined resulting in baseline (pre) versus testosterone treatment (post, regardless of mode of treatment) comparisons. In human subjects, pre-post testosterone treatment differences were compared using paired *t* tests. Linear regression analysis was used to examine the relationship between NIK and serum total testosterone. In cell culture experiments, two-way repeated measures analysis of variance (ANOVA) analyses were performed to evaluate time and treatment effects. This was followed by Tukey's post hoc test to determine pair-wise significance if the ANOVA test was significant. In all cases, $P < .05$ was considered significant.

RESULTS

To assess if NIK levels increase with aging in the human population, vastus lateralis muscle was sampled from younger and older subjects, then assayed for NIK expression using quantitative real time (qRT)–PCR. Figure 1 shows delta CT for the two groups (a lower delta CT means higher mRNA expression), and a significant increase in NIK mRNA expression is evident in the older versus younger subjects. These results are consistent with our previous report of

increased NIK in skeletal muscle sampled from obese, insulin-resistant human subjects (42).

We next assessed effects of testosterone therapy on skeletal muscle NIK expression. After baseline sampling of plasma and skeletal muscle tissue, we administered testosterone as either a single intramuscular injection (testosterone enanthate, 100 mg) or as a gel applied daily to the skin (AndroGel, 10 g/d). After 7 days, plasma and skeletal muscle tissue were again collected for a before and after comparison. Protein was extracted from skeletal muscle of six different subjects before (Figure 2A, *black bars*) and after (Figure 2A, *gray bars)* 1 week of testosterone intervention. Quantification of the immunoblots suggests that testosterone treatment can decrease skeletal muscle NIK levels within 7 days in most subjects. Figure 2B is a bar graph compilation of all western blots.

Even more striking is our discovery of an inverse relationship between serum testosterone concentrations and skeletal muscle NIK expression in men with low-normal endogenous testosterone (ie, less than 500 ng/dL). Figure 3 (*upper panel*) shows a significant negative association for basal plasma testosterone and NIK protein levels, whereas the lower panel shows a negative correlation between the change in plasma testosterone levels and NIK protein expression after 1 week of treatment, with an R2 value of 0.2278 and correlation coefficient of -0.477. Regression analysis did not reach statistical significance due to the small sample size. These data indicate that as little as 7 days of testosterone treatment can decrease skeletal muscle NIK levels.

To test if these in vivo results could be reproduced in vitro, we used differentiated C2C12 cells to show a substantial increase in NIK protein expression 6 hours after initiating methylprednisolone treatment (0.5 μg/mL) (Figure 4). Peak levels were reached at 24 hours versus dimethyl sulfoxide (DMSO)–treated controls. This is intriguing data because methylprednisolone is given to patients as an anti-inflammatory agent but results in increased levels of NIK within skeletal muscle. Differentiated C2C12 cells treated with methylprednisolone for various time intervals also resulted in time-dependent increases in the skeletal muscle–specific atrophy marker, atrogin-1 (Figure 4C).

To test effects of testosterone in vitro, we exposed differentiated primary human skeletal myoblasts to methylprednisolone (0.5 μg/mL) for 24 hours in tissue culture. Testosterone was then added at 1 μM concentration in the presence of glucocorticoid for indicated times as shown in Figure 5A. NIK mRNA expression levels were increased 2.5-fold after methylprednisolone treatment ($^*P < .01$ vs untreated cells), and this methylprednisolone effect was rapidly reversed to

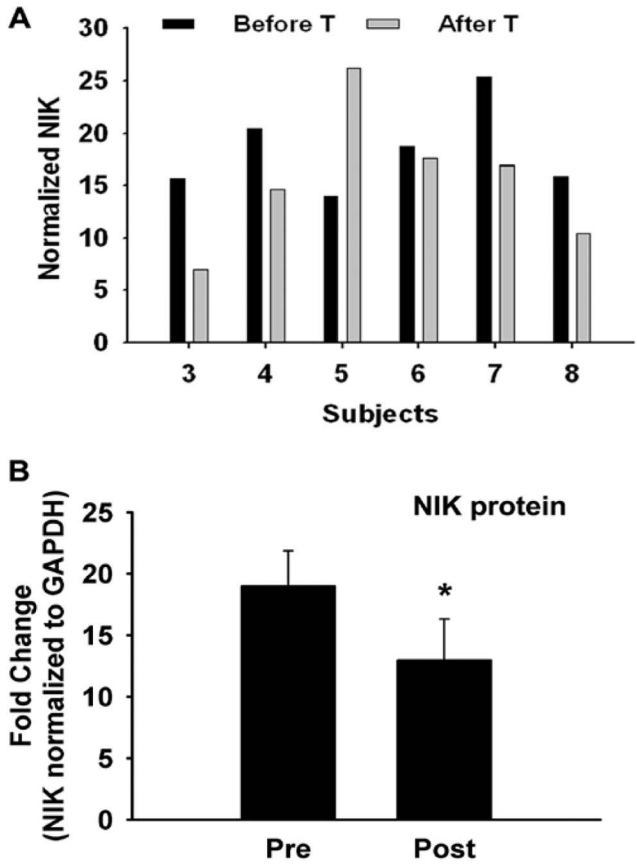

FIG. 2. Decrease in skeletal muscle NIK message and protein levels after testosterone therapy in elderly subjects. After collection of baseline plasma and skeletal muscle biopsy specimens (before, *black bars*), subjects were given testosterone treatment as either a single intramuscular injection (100 mg) or as a gel to be applied daily to the skin (10 g/d). On day 7, another set of samples was collected (after T, *gray bars*). **(A)** Quantification of the individual before and after western blots. **(B)** Quantification of the immunoblots indicates that 1 week of testosterone intervention reduces skeletal muscle NIK levels.

control levels by testosterone ($^{\dagger}P < .01$ vs methylprednisolone-treated cells). The observation that testosterone completely normalized glucocorticoid-induced increases in NIK levels by the earliest time point sampled (2 hours) was a striking observation. We also showed that testosterone suppressed methylprednisolone-induced NIK protein expression in primary skeletal muscle cells (Figure 5B). A similar effect of testosterone on MuRF1 protein expression was observed.

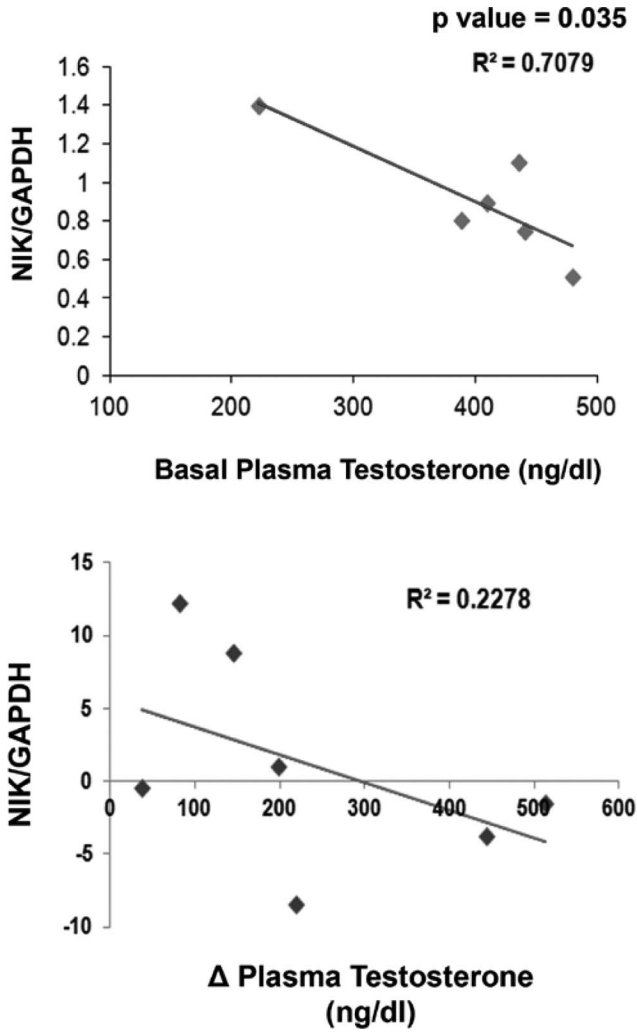

FIG. 3. A negative correlation between plasma testosterone and skeletal muscle NIK protein expression. Upper panel shows a significant ($P < .035$) negative association between basal plasma testosterone and NIK protein levels; the lower panel shows a negative correlation between the change in plasma testosterone levels and NIK protein expression after 1 week of treatment.

DISCUSSION

Increased NIK levels have been measured in skeletal muscle of patients with a variety of disorders such as multiple myeloma (12) and metabolic syndrome (42). Although virtually nothing is known about

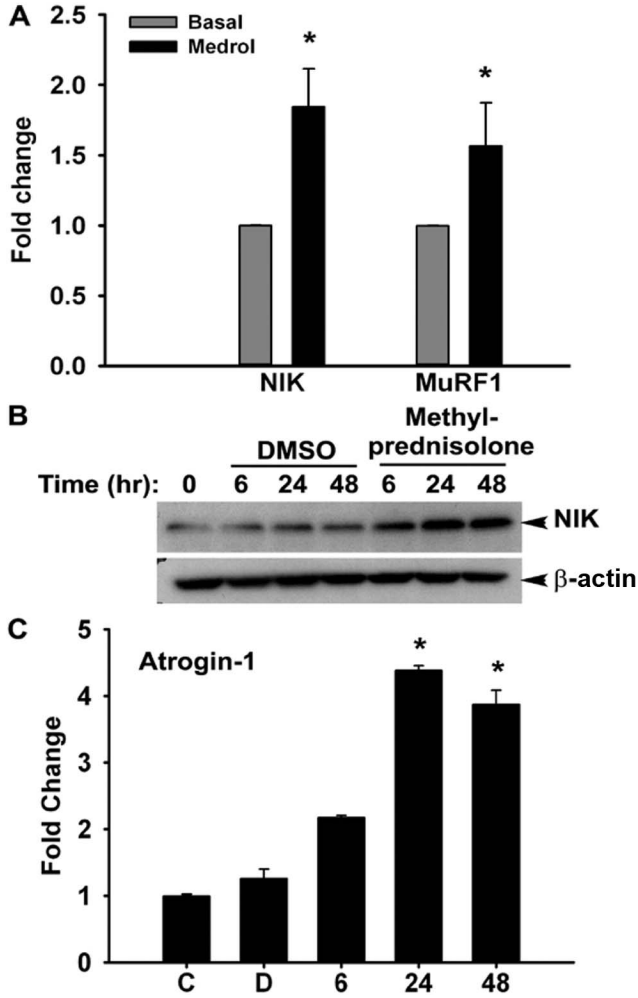

FIG. 4. **(A)** Skeletal muscle biopsy specimens were collected from the vastus lateralis of healthy human subjects (n = 3) for basal measurement of NIK and MuRF 1 expression, followed by a 6-day, graded dosing regimen of methylprednisolone (24, 20, 16, 12, 8, and 4 mg on days 1 through 6, respectively). A second biopsy was obtained at the end of the glucocorticoid treatment. NIK and MuRF1 mRNA levels were quantified with RT-PCR. (**B**) Methylprednisolone-induced increases in NIK protein also were measured with immunoblotting in differentiated C2C12 cells 6 hours after initiating methylprednisolone treatment (0.5 μg/mL). Peak levels were reached at 24 hours versus DMSO-treated controls. **(C)** Methylprednisolone increases Atrogin-1 mRNA expression. Differentiated C2C12 cells were either treated with DMSO (D, vehicle control) or methylprednisolone for 6, 24, and 48 hours, respectively. (C) Shows untreated cells. Data represent fold change expression as compared to untreated cells. *$P < .05$ significantly different than untreated cells.

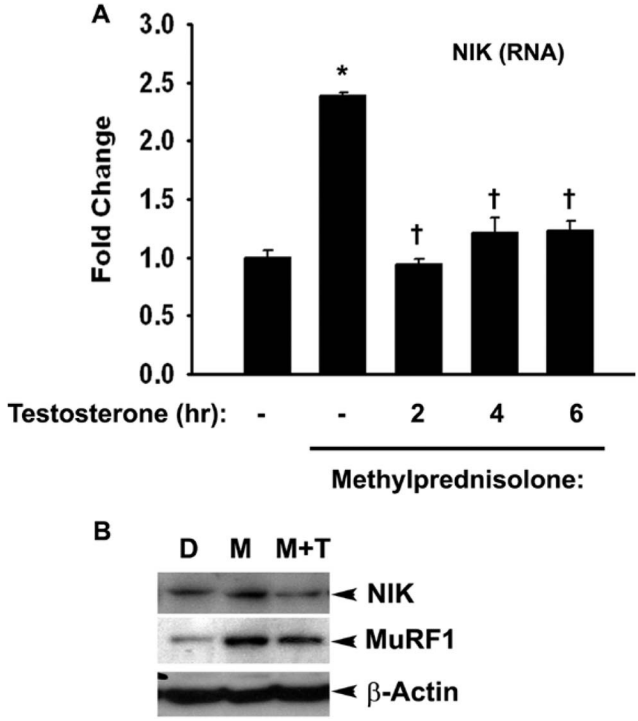

FIG. 5. **(A)** Differentiated primary human skeletal myoblasts were exposed to methylprednisolone (0.5 μg/mL) for 24 hours in tissue culture. Testosterone was then added at 1 μM concentration in the presence of glucocorticoid for indicated times. NIK mRNA expression levels were measured by qRT-PCR using GAPDH as housekeeping gene for data normalization. **(B)** Methylprednisolone-induced expression of NIK protein is suppressed by testosterone. Differentiated primary cultures of human skeletal myoblasts were treated with methylprednisolone, (M) (0.5 μg/mL) for 24 hours. Testosterone (T) was added at 1 μM concentration for indicated times. DMSO (D) serves as vehicle control.

NIK regulation in skeletal muscle cells, how NIK levels are regulated in other cells has been well characterized. NIK is an activating kinase that is constantly synthesized but tightly regulated at low basal levels in cells via binding to tumor necrosis factor receptor-associated factors (TRAFs). In the unstressed cell, TRAF3 recruits NIK to a complex containing TRAF2 and cIAP1/2, and NIK undergoes ubiquitination by cIAP1/2 resulting in rapid proteosomal degradation. In the presence of NF-κB noncanonical pathway stimuli, cIAP1/2 ubiquitinates and degrades TRAF3, releasing NIK from its negative regulation by TRAF3, leading to its stabilization and accumulation in cells (43, 44). An increased level of NIK causes its activation, presumably by autophos-

phorylation, and high intracellular NIK levels can activate the NF-κB pathway, thus contributing to catabolic signaling. It remains to be determined whether chronic upregulation of skeletal muscle NIK is involved in the onset or progression of sarcopenia and other wasting conditions.

Here, we have shown that aging is associated with increased basal levels of skeletal muscle NIK content when compared to young muscle, and that skeletal muscle NIK content can be decreased in older men receiving testosterone supplementation for 1 week. Further support that testosterone is involved in the downregulation of noncanonical NF-κB signaling is our recent published finding that testosterone therapy in combination with oral protein supplementation reduced expression of p52 and RelB in skeletal muscle of a cervical cancer patient, despite persistent inflammation and upregulation of p50 and RelA (45).

Using tissue culture, we have shown that testosterone directly inhibits the upregulation of NIK in primary human skeletal muscle and C2C12 cells in response to a glucocorticoid stimulus that increases NIK levels. These results collectively suggest a protective role for testosterone against NIK-mediated signaling and could provide a mechanism behind its anti-inflammatory and anti-catabolic properties. To the best of our knowledge, this is the first report suggesting that testosterone may work to inhibit the accumulation of NIK in skeletal muscle. Our observation that testosterone preferentially affects the noncanonical NF-κB signaling axis via modulation of NIK is a highly novel finding in the field of muscle biology. This is significant because NIK is well-known as a key upstream regulator of the noncanonical NF-κB·RelB/p52 signaling cascade, a pathway responsive to induction by glucocorticoids (46–49) and identified in the regulation of skeletal myogenesis (50, 51).

Sarcopenia affects approximately 5% of people at the age of 65 years and nearly 50% of people aged 80 years and older (52–54). Thus, an effective therapy to safely stimulate skeletal muscle anabolism and suppress catabolism is needed. The age-related differences in skeletal muscle NIK levels in both men and women in the present data suggest that, in addition to testosterone, other regulatory factors likely contribute to changes in skeletal muscle NIK regulation. Premenopausal women experience decreases in androgens, including testosterone, with increased age (55). However, endogenous testosterone levels in women rely on production by the ovary and adrenal cortex which is an order of magnitude lower than that derived from the gonads in men at any age. Testosterone administration clearly attenuated NIK levels in

older men and testosterone directly repressed NIK expression in vitro but we cannot exclude the possibility that related hormones, such as DHEA, dihydrotestosterone (DHT) or estrogen, regulate NIK equally or more potently in younger men and women.

Although the molecular mechanisms responsible are not known, we have shown that methylprednisolone induces NIK mRNA expression in human skeletal muscle in tissue culture, and that this is associated with an increase in MuRF1 mRNA expression. Here, MuRF1 is used as a marker of skeletal muscle catabolism. Our in vitro experiments also show that induction of NIK expression in human primary skeletal muscle cells coincides with increased expression of the catabolic signaling protein atrogin-1. Finally, the clinical data, supported by data obtained from the in vitro cell culture experiments, show that testosterone attenuates the upregulation of NIK in skeletal muscle cells.

In summary, our results indicate that increased age is associated with increased skeletal muscle NIK levels in men and women. We showed that as little as 1 week of testosterone treatment in men with endogenous testosterone in the low-normal range resulted in increased testosterone levels and that this correlated with decreased skeletal muscle NIK levels. Future experiments are warranted to explore the molecular mechanisms involved in the modulation of NIK levels by anabolic/anti-catabolic agents, to investigate the role of NIK signaling in protein catabolism and skeletal muscle wasting, and to elucidate the possible clinical implications associated with altered skeletal muscle NIK signaling.

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REFERENCES

1. Fry CS, Drummond MJ, Glynn EL, et al. Skeletal muscle autophagy and protein breakdown following resistance exercise are similar in younger and older adults. *J Gerontol A Biol Sci Med Sci* 2012;68:599–607.
2. Bruunsgaard H, Andersen-Ranberg K, Hjelmborg J, et al. Elevated levels of tumor necrosis factor alpha and mortality in centenarians. *Am J Med* 2003;115:278–83.
3. Woods JA, Wilund KR, Martin SA, et al. Exercise, inflammation and aging. *Aging Dis* 2012;3:130–40.
4. Aguilera G. HPA axis responsiveness to stress: implications for healthy aging. *Exp*

Gerontol 2010;46:90–5.
5. Born J, Ditschuneit I, Schreiber M, et al. Effects of age and gender on pituitary-adrenocortical responsiveness in humans. *Eur J Endocrinol* 1995;132:705–11.
6. Garrido P. Aging and stress: past hypotheses, present approaches and perspectives. *Aging Dis* 2012;2:80–99.
7. Seidman SN. Androgens and the aging male. *Psychopharmacol Bull* 2007;40:205–18.
8. Valenti G. Aging as an allostasis condition of hormones secretion: summing up the endocrine data from the inChianti study. *Acta Biomed* 2010;81(suppl 1):9–14.
9. Kumar A, Bhatnagar S, Paul PK. TWEAK and TRAF6 regulate skeletal muscle atrophy. *Curr Opin Clin Nutr Metab Care* 2012;15:233–9.
10. Yamaki T, Wu CL, Gustin M, et al. Rel A/p65 is required for cytokine-induced myotube atrophy. *Am J Physiol Cell Physiol* 2012;303:C135–42.
11. Starkey JM, Haidacher SJ, LeJeune WS, et al. Diabetes-induced activation of canonical and noncanonical nuclear factor-kappaB pathways in renal cortex. *Diabetes* 2006;55:1252–9.
12. Demchenko YN, Glebov OK, Zingone A, et al. Classical and/or alternative NF-kappaB pathway activation in multiple myeloma. *Blood* 2010;115:3541–52.
13. Aya K, Alhawagri M, Hagen-Stapleton A, et al. NF-(kappa)B-inducing kinase controls lymphocyte and osteoclast activities in inflammatory arthritis. *J Clin Invest* 2005;115:1848–54.
14. Brunton SA, Sadovsky R. Late-onset male hypogonadism and testosterone replacement therapy in primary care. *J Family Practice* 2010;59:S1–8.
15. Kaltenboeck A, Foster S, Ivanova J, et al. The direct and indirect costs among U.S. privately insured employees with hypogonadism. *J Sex Med* 2012;9:2438–47.
16. Baillargeon J, Urban RJ, Ottenbacher KJ, et al. Trends in androgen prescribing in the United States, 2001 to 2011. *JAMA* 2013;173:1465–6.
17. Dillon EL, Durham WJ, Urban RJ, et al. Hormone treatment and muscle anabolism during aging: androgens. *Clin Nutr* 2010;29:697–700.
18. Sheffield-Moore M. Androgens and the control of skeletal muscle protein synthesis. *Ann Med* 2000;32:181–6.
19. Sheffield-Moore M, Casperson S, Urban RJ. Androgens and lean body mass in the aging male. In: Lunenfeld B et al, eds. *Textbook of Men's Health and Aging. 2nd ed*. Boca Raton, Florida: CRC Press; 2007:307–12.
20. Sheffield-Moore M, Dillon EL, Casperson SL, et al. A randomized pilot study of monthly cycled testosterone replacement or continuous testosterone replacement versus placebo in older men. *J Clin Endocrinol Metab* 2011;11:E1831–7.
21. Sheffield-Moore M, Paddon-Jones D, Casperson SL, et al. Androgen therapy induces muscle protein anabolism in older women. *J Clin Endocrinol Metab* 2006;91:3844–9.
22. Sheffield-Moore M, Urban RJ, Wolf SE, et al. Short-term oxandrolone administration stimulates net muscle protein synthesis in young men. *J Clin Endocrinol Metab* 1999;84:2705–11.
23. Sheffield-Moore M, Wolfe RR, Gore DC, et al. Combined effects of hyperaminoacidemia and oxandrolone on skeletal muscle protein synthesis. *Am J Physiol Endocrinol Metab* 2000;278:E273–9.
24. Bhasin S. Testosterone supplementation for aging-associated sarcopenia. *J Gerontol A Biol Sci Med* 2003;58:1002–8.
25. Bhasin S, Calof OM, Storer TW, et al. Drug insight: testosterone and selective androgen receptor modulators as anabolic therapies for chronic illness and aging. *Nat Clin Pract Endocrinol Metab* 2006;2:146–59.
26. Bhasin S, Cunningham GR, Hayes FJ, et al. Testosterone therapy in adult men with androgen deficiency syndromes: an endocrine society clinical practice guideline.

J Clin Endocrinol Metab 2006;91:1995–2010.
27. Bhasin S, Storer TW, Berman N, et al. Testosterone replacement increases fat-free mass and muscle size in hypogonadal men. *J Clin Endocrinol Metab* 1997;82:407–13.
28. Bhasin S, Woodhouse L, Storer TW. Proof of the effect of testosterone on skeletal muscle. *J Endocrinol* 2001;170:27–38.
29. Sinha-Hikim I, Cornford M, Gaytan H, et al. Effects of testosterone supplementation on skeletal muscle fiber hypertrophy and satellite cells in community-dwelling older men. *J Clin Endocrinol Metab* 2006;91:3024–33.
30. Ferrando AA, Sheffield-Moore M, Yeckel CW, et al. Testosterone administration to older men improves muscle function: molecular and physiological mechanisms. *Am J Physiol Endocrinol Metab* 2002;282:E601–7.
31. Sheffield-Moore M, Dillon EL, Casperson SL, et al. A randomized pilot study of monthly cycled testosterone replacement or continuous testosterone replacement versus placebo in older men. *J Clin Endocrinol Metab* 2011;96:E1831–7.
32. Iwasaki Y, Asai M, Yoshida M, et al. Dehydroepiandrosterone-sulfate inhibits nuclear factor-kappaB-dependent transcription in hepatocytes, possibly through antioxidant effect. *J Clin Endocrinol Metab* 2004;89:3449–54.
33. Sculthorpe N, Solomon AM, Sinanan AC, et al. Androgens affect myogenesis in vitro and increase local IGF-1 expression. *Med Sci Sports Exercise* 2012;44:610–5.
34. Qin W, Pan J, Wu Y, et al. Protection against dexamethasone-induced muscle atrophy is related to modulation by testosterone of FOXO1 and PGC-1alpha. *Biochem Biophys Res Comm* 2010;403:473–8.
35. Wu Y, Bauman WA, Blitzer RD, et al. Testosterone-induced hypertrophy of L6 myoblasts is dependent upon Erk and mTOR. *Biochem Biophys Res Comm* 2010;400:679–83.
36. Kadi F. Cellular and molecular mechanisms responsible for the action of testosterone on human skeletal muscle. A basis for illegal performance enhancement. *Br J Pharmacol* 2008;154:522–8.
37. Pires-Oliveira M, Maragno AL, Parreiras-e-Silva LT, et al. Testosterone represses ubiquitin ligases atrogin-1 and Murf-1 expression in an androgen-sensitive rat skeletal muscle in vivo. *J Appl Physiol* 2010;108:266–73.
38. Bergstrom J. Percutaneous needle biopsy of skeletal muscle in physiological and clinical research. *Scand J Clin Lab Invest* 1975;35:609–16.
39. Pavlath GK, Gussoni E. Human myoblasts and muscle-derived SP cells. *Methods Mol Med* 2005;107:97–110.
40. Thompson MG, Thom A, Partridge K, et al. Stimulation of myofibrillar protein degradation and expression of mRNA encoding the ubiquitin-proteasome system in C(2)C(12) myotubes by dexamethasone: effect of the proteasome inhibitor MG-132. *J Cell Physiol* 1999;181:455–61.
41. Zhao W, Pan J, Zhao Z, et al. Testosterone protects against dexamethasone-induced muscle atrophy, protein degradation and MAFbx upregulation. *J Steroid Biochem Mol Biol* 2008;110:125–9.
42. Choudhary S, Sinha S, Zhao Y, et al. NF-kappaB-inducing kinase (NIK) mediates skeletal muscle insulin resistance: blockade by adiponectin. *Endocrinology* 2011;152:3622–7.
43. Vallabhapurapu S, Matsuzawa A, Zhang W, et al. Nonredundant and complementary functions of TRAF2 and TRAF3 in a ubiquitination cascade that activates NIK-dependent alternative NF-kappaB signaling. *Nat Immunol* 2008;9:1364–70.
44. Zarnegar BJ, Wang Y, Mahoney DJ, et al. Noncanonical NF-kappaB activation requires coordinated assembly of a regulatory complex of the adaptors cIAP1, cIAP2,

TRAF2 and TRAF3 and the kinase NIK. *Nat Immunol* 2008;9:1371–8.
45. Dillon EL, Basra G, Horstman AM, et al. Cancer cachexia and anabolic interventions: a case report. *J Cachexia Sarcopenia Muscle* 2012;3:253–63.
46. Castro-Caldas M, Mendes AF, Carvalho AP, et al. Dexamethasone prevents interleukin-1beta-induced nuclear factor-kappaB activation by upregulating IkappaB-alpha synthesis, in lymphoblastic cells. *Mediators Inflamm* 2003;12:37–46.
47. Ivanov VN, Deng G, Podack ER, et al. Pleiotropic effects of Bcl-2 on transcription factors in T cells: potential role of NF-kappa B p50–p50 for the anti-apoptotic function of Bcl-2. *Int Immunol* 1995;7:1709–20.
48. Solan NJ, Miyoshi H, Carmona EM, et al. RelB cellular regulation and transcriptional activity are regulated by p100. *J Biol Chem* 2002;277:1405–18.
49. Wang B, Parobchak N, Rosen T. RelB/NF-kappaB2 regulates corticotropin-releasing hormone in the human placenta. *Mol Endocrinol* 2012;26:1356–69.
50. Bakkar N, Guttridge DC. NF-kappaB signaling: a tale of two pathways in skeletal myogenesis. *Physiol Rev* 2010;90:495–511.
51. Bakkar N, Wang J, Ladner KJ, et al. IKK/NF-kappaB regulates skeletal myogenesis via a signaling switch to inhibit differentiation and promote mitochondrial biogenesis. *J Cell Biol* 2008;180:787–802.
52. Morley JE. Sarcopenia in the elderly. *Fam Pract* 2012;29(suppl 1):i44–i8.
53. Morley JE. Sarcopenia: diagnosis and treatment. *J Nutr Health Aging* 2008;12:452–6.
54. Baumgartner RN, Koehler KM, Gallagher D, et al. Epidemiology of sarcopenia among the elderly in New Mexico. *Am J Epidemiol* 1998;147:755–63.
55. Davison SL, Bell R, Donath S, et al. Androgen levels in adult females: changes with age, menopause, and oophorectomy. *J Clin Endocrinol Metab* 2005;90:3847–53.

DISCUSSION

Spivak, Baltimore: It gives us older men hope here. As a hematologist, I constantly see men given testosterone as replacement therapy — and as their physicians always say, "But it's just replacement therapy" — who come in with extremely high hematocrits. And testosterone will squeeze the plasma volume, so a lot of these men do not have an elevated red cell mass. Actually, they have plasma volume contraction, which theoretically increases blood viscosity. And I am curious: in your studies, did you have a chance to measure hematocrits just to see if there is there is any change there?

Urban, Galveston: Yes. We have done that in all of our studies and there is a change. Any time you use IM injections, it is probably the number one problem that we get with testosterone. So yes, it is a real entity, and the gels cause a little less problem than the injections. The injections primarily are the big culprit. But any time that I am replacing a patient with testosterone, I always do a hematocrit before I start, and then I check every 6 months, because that is the biggest limiting factor in the use of testosterone. I agree with you wholeheartedly.

Spivak, Baltimore: I guess there is an anecdote and that would be if you just bleed the patients, they expand their plasma volume extremely rapidly. And it probably wouldn't take bleeding that often to keep the hematocrits down where they should be.

Urban, Galveston: I think also the testosterone stimulates the red blood cell production as well though, so you've got two factors working there.

Spivak, Baltimore: It's interesting. It does, but not as much as . . . .

Urban, Galveston: . . .as the volume contraction. That's good to know.

Dupont, Houston: Randy, very nice presentation and study. What was the relationship between baseline testosterone serum levels in these patients and benefit of exoge-

nous testosterone therapy? And again, looking through Mary Shelley's eyes at your work, are there any negative things to this — cardiovascular effects or prostate enlargement in these patients — that might be things we should worry about?

Urban, Galveston: Thanks Burt. We don't know the answer about prostate cancer. There is currently an NIH study that's ongoing, a multi-site study, where it's kind of like the old WHO trial where they've given testosterone to a group of actually hypogonadal older men, and they are trying to see if they are going to see an increased incidence of prostate cancer. So that data isn't out there. Everyone thinks that it's probably the case. Of course, that's an androgen-sensitive organ. So when I start testosterone in my older men, I tell them that that's a real risk that you have to worry about. The cardiovascular side of it is really a mixed bag, and I'm sure there are some cardiologists here who know much more about this than me. Of course, it lowers HDL, and so there is the risk of vascular disease. And Shalender Bhasin had published the New England Journal of Medicine article where he gave testosterone to a group of older, very ill men, and he increased their risk of cardiovascular disease. So certainly that is a huge problem that you have to be very aware of. But it does a lot of other things as well. It also lowers vascular resistance, so there could be some positives to it. And there are data that I didn't show you here, but we are actually now starting in cachectic cancer patients, to do echoes to begin to look at the function of the heart muscle and we've got some preliminary data which shows that heart is a muscle too! We've never really focused on the cardiac muscle, so we are beginning to see an improvement in performance in that heart muscle. So it's not a simple answer, but I think it's one that you just really have to discuss with your patients.

Dupont, Houston: What about serum levels of testosterone?

Urban, Galveston: We've never done large enough studies where we've really been able to do that. You know, these studies, which are done with stable isotope infusions are usually done with eight men so we really don't have the statistical power to say that if a patient was low initially and we raised his level, and we saw a significant difference.

Pasche, Birmingham: As an oncologist, I was quite impressed by your data on the cancer patient moving around much more. In oncology, we know that performance status, which is essentially how much you are able to carry on, trumps anything in terms of prognosis with respect to longevity of patients. Did you see any difference in outcome? Are you planning any trial where you are going to treat the patient that may be less terminal to try to find out whether you can impact the long-term outcome of these patients?

Urban, Galveston: We haven't seen any difference in outcome, but our numbers are very small: Only 13–14 patients so far. And yes, we think that this is something that we ought to now move to a younger, less involved group. This was our beginning way to see if we can actually see something with this group and we'd like to try a group that isn't quite as end-stage. Thank you.

Mitch, Baylor: I was curious if you've looked a bit more proximally, because there have been reports, experimentally, that myostatin inhibition will actually prolong life in animal models of cancer, and we were able to show the same thing in chronic kidney disease.

Urban, Galveston: No we haven't, Bill. That's a good thought, but we've just been sort of focusing on this catabolic pathway right now.

Dale, Seattle: How much of the effect is on proliferation versus cell survival or hypertrophy?

Urban, Galveston: Well, you're really asking the question of synthesis versus breakdown. So testosterone is an interesting compound because it stimulates both. So we know that there is one side of testosterone that's actually stimulating the satellite cells

to go into causing hypertrophy and increase of muscle mass. So it does that, and this is not really what these studies are focusing on. They are focusing on its other thing, which is its ability to inhibit the catabolism. We haven't been able to carefully balance between the two to see which one is more prevalent than the other.

Hochberg, Baltimore: So you mentioned that you did muscle biopsies in these subjects. Have you looked at the amount of small vessels as to whether or not there is an increase? And also, have you looked at any kind of studies of mitochondrial function with regard to oxidative stress?

Urban, Galveston: We have not. Good thought.

Berger, Cleveland: From the oncology viewpoint, the problem with performance status is particularly serious in older African American women with breast cancer who have the worst functional decline early in their disease, and this correlates really very clearly with prognosis. So the question is, have you looked at any difference in racial effect of the testosterone, and have you looked at women? That's most important.

Urban, Galveston: No. These are just very preliminary, but those are great points.